BRIEF COMMUNICATION OPEN

C1s protects cutaneous squamous carcinoma cells from TRAIL-induced apoptosis

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Cutaneous squamous cell carcinoma (cSCC) is the most common metastatic skin cancer. The prognosis of the metastatic cSCC is poor, and there are no established biomarkers to predict metastasis risk, nor specific targeted therapies for advanced or metastatic cSCC. Previous studies have demonstrated that the complement serine proteinase C1s promotes cSCC growth both in culture and in vivo by modulating apoptotic signaling. Here, we investigated the mechanistic role of C1s in regulating apoptosis by examining its impact on cell surface proteome of cSCC cells. Mass spectrometric analysis of cell surface proteins following silencing of C1s identified TRAIL receptor 1 (DR4) as a candidate target, showing increased accumulation at the cell surface. This finding was validated using cell surface biotinylation and western blot analysis in both siRNA-mediated C1s knockdown and CRISPR/Cas9-generated C1s knockout cells. Functionally, high endogenous levels or forced overexpression of C1s conferred resistance to TRAIL-induced apoptosis in cSCC cells, whereas reduced C1s levels sensitized cells to apoptotic signaling. These findings suggest that upregulation of complement C1s in cSCC not only contributes to tumor progression but also serves as a protective mechanism against TRAIL-induced apoptosis, highlighting its potential as a therapeutic target and biomarker in aggressive cSCC.

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INTRODUCTION

Keratinocyte-derived non-melanoma skin cancers (NMSCs) are the most common malignancies in humans, with a rising global incidence [1]. Among these, cutaneous squamous cell carcinoma (cSCC) is the most prevalent metastatic skin cancer and carries high mortality rates in the advanced stages. The estimated metastasis rate for primary cSCC is 3–5%, and the prognosis for metastatic disease is poor, with less than 30% 3-year survival [2, 3]. Major risk factors for cSCC development and progression include chronic exposure to solar ultraviolet radiation (UVR), immunosuppression, chronic inflammation, and chronic dermal ulcers [4]. UV-induced cSCC typically progresses from premalignant lesion, actinic keratosis, to cSCC in situ (Bowen's disease) and ultimately to invasive and metastatic cSCC [4]. Tumorigenesis in cSCC is driven by high mutational burden, largely due to cumulative UV exposure. Early events in cSCC pathogenesis include the mutational inactivation of tumor suppressor gene *TP53* and *NOTCH1* genes in epidermal keratinocytes [5–7]. Other commonly mutated genes in cSCC include *HRAS*, *CDKN2A*, and *CASP8* [7–9]. In addition, alterations in the tumor microenvironment are crucial for cSCC development and progression [10–12].

The complement system, a key component of the innate immune system, can be activated through classical, lectin, or alternative pathways, which all converge on the cleavage of C3, leading to activation of terminal pathway and formation of the lytic membrane attack complex [13]. The serine proteinase C1s is a critical component of the classical pathway initiating C1 complex ($C1qr_2s_2$). Upon binding of C1q to a target molecule, C1r undergoes autocatalytic activation and, in turn, activates C1s [14]. Emerging evidence suggests that components of the C1 complex, particularly C1r and C1s, contribute to cancer progression through non-canonical, complement-independent mechanisms [15–18]. In previous studies, we observed significant upregulation of C1r and C1s in cSCC cell lines in culture and tumors in vivo. Furthermore, both C1r and C1s were shown to promote cSCC growth by regulating apoptosis [15, 16, 19]. Here, we investigated the mechanistic role of the serine proteinase C1s in the progression of cSCC. These findings show that C1s protects cSCC cells from TRAIL-mediated apoptosis and suggest that targeting C1s could enhance the efficacy of TRAIL-based therapies.

RESULTS

Mass spectrometric analysis of cSCC cell surface proteins following C1s silencing

To investigate the effect of the serine proteinase C1s on cell surface proteome of cSCC cells, we performed cell surface biotinylation followed by mass spectrometric analysis after C1s knockdown. The most significantly altered extracellular cell surface-associated proteins are presented as volcano plot (Fig. 1A). This analysis specifically focused on identifying upregulated extracellular domains of cell surface proteins, aiming to uncover molecules enriched on the cell surface in response to C1s silencing.

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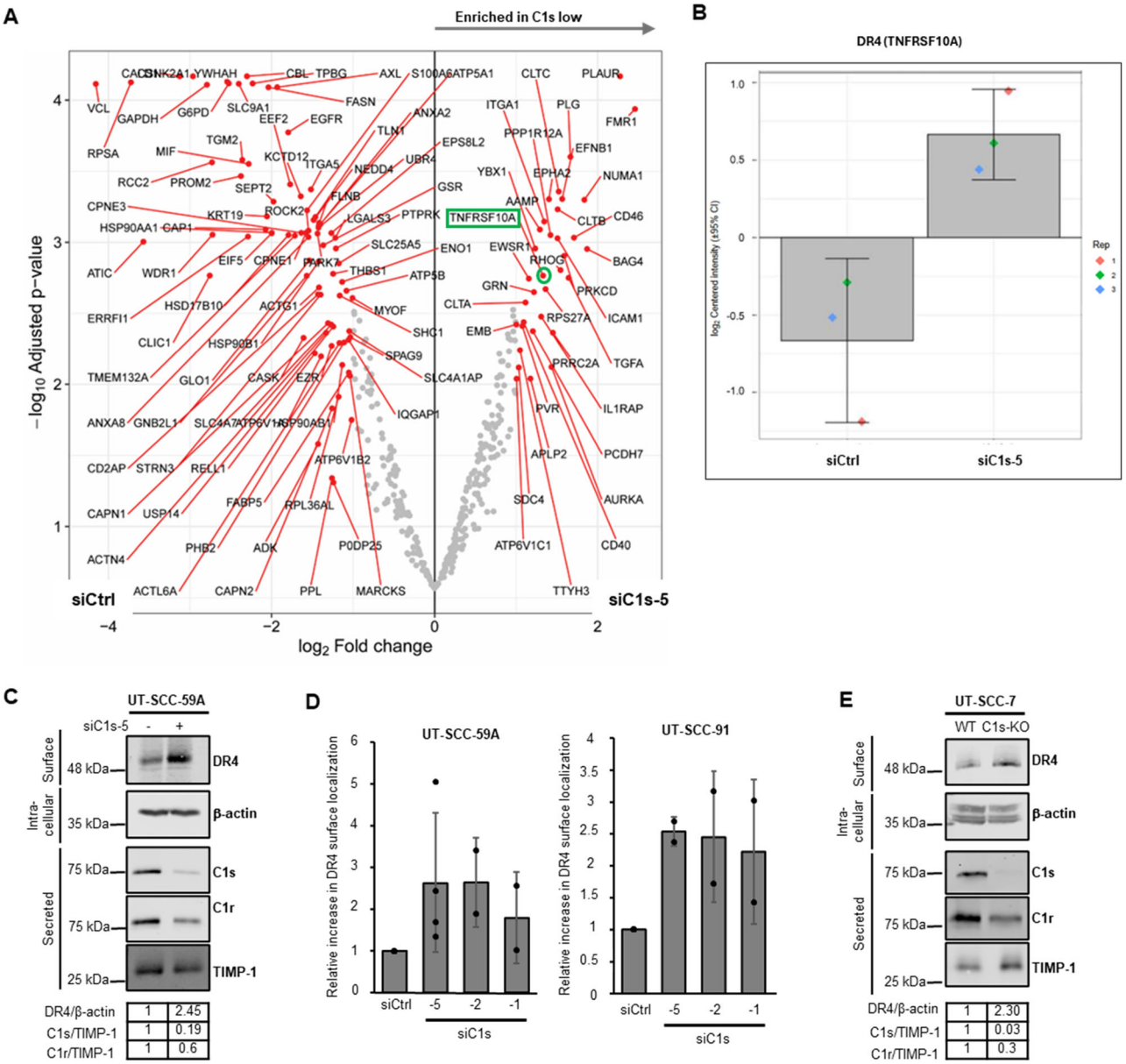

Fig. 1 DR4 is enriched at the cSCC cell surface following C1s silencing. A, **B** cSCC cells (UT-SCC-59A) were transfected with C1s-specific siRNAs or control siRNA (120 nM). After 72 h, cell surface proteins were biotinylated, and the biotinylated molecules from three independent replicates were analyzed by mass spectrometry. **A** The volcano plot shows the most significantly regulated cell surface proteins in cSCC cells following C1s knockdown ($n = 3$). DR4 (TNFRSF10A) is represented in green. **B** The box blot illustrating the significant enrichment of DR4 at the cell surface in C1s-silenced cSCC cells in three independent replicates (rep). **C**, **D** Western blot analysis of biotinylated cell surface proteins pulled down with streptavidin-coated agarose beads following C1s silencing. DR4 levels were assessed in the pulldown fraction, while β-actin in the supernatant served as a control for sample loading and cytosolic content. The levels of secreted C1s and C1r in the conditioned media were compared with TIMP-1, a ubiquitously expressed and secreted protein. **D** Summary of cell surface protein pulldown assays in cSCC cell lines transfected with three distinct C1s-targeting siRNAs. Quantification of biotinylation Western blots is shown. Data are presented as mean ± s.d. with individual data points. **E** Western blot analysis of biotinylated cell surface proteins pulled down using streptavidin-coated agarose beads, comparing UT-SCC-7 wild-type (WT) cells and a CRISPR-Cas9-mediated C1s knockout (C1s-KO) cell population from three independent pooled clones. Levels of secreted C1s and C1r in conditioned media were compared with TIMP-1.

DR4 accumulates on the cell surface of cSCC cells following C1s silencing

Given the previously shown role of C1s in regulating apoptosis in cSCC cells it was notable that mass spectrometric analysis identified death receptor 4 (DR4; also known as TRAIL-R1, TNFRSF10A) as one of the most enriched cell surface proteins following C1s silencing (Fig. 1A, B). RNA sequencing data from our previous study [16] indicated that DR4 mRNA levels remained unchanged in C1s knockdown cells (Supplementary Table S1), suggesting post-transcriptional regulation. Western blot analysis confirmed that DR4 was enriched in the membrane fraction of cSCC cells after C1s silencing with specific siRNAs (Fig. 1C, D; Supplementary Fig. S1), as well as in C1s knockout (C1s-KO) cells (Fig. 1E).

DR4 is expressed on the surface of cSCC cell lines

A comparative analysis of the death receptor expression on the surface of SCC cell lines revealed that DR4 is abundantly expressed

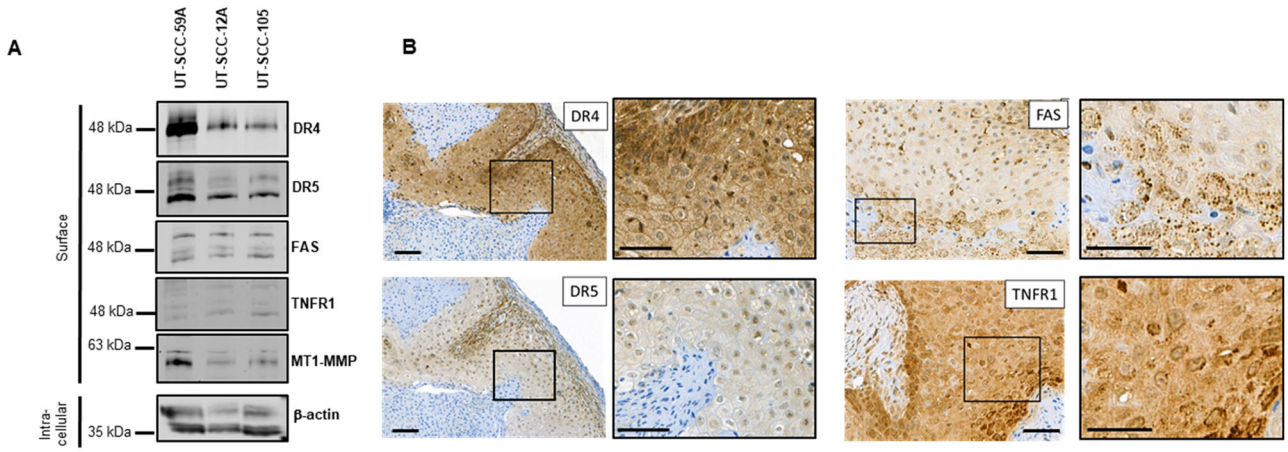

Fig. 2 DR4 is abundantly expressed on the surface of cSCC cells in culture and in vivo. A Western blot analysis of biotinylated cell surface proteins pulled down using streptavidin-coated agarose beads. The levels of cell surface proteins were assessed from the pulldown fraction, with MT1-MMP and β-actin in the supernatant serving as sample and quality controls. The analysis was performed using the cSCC cell lines indicated. **B** cSCC cells (UT-SCC-91; 7×10^6) were injected subcutaneously into the dorsal skin of severe combined immunodeficient mice. Xenograft tumors were excised after 16 days and subjected to immunohistochemical staining for DR4, DR5, FAS, and TNFR1. Mayer's hematoxylin was used as a counterstain. Representative images of stainings are shown with higher magnification views of the images shown on the right. Scale bar = 100 µm.

by cSCC cells, and DR5 and FAS were also detectable by western blotting (Fig. 2A; Supplementary Fig. S2). In contrast, TNFR1 was not detected at the cell surface. Immunohistochemical (IHC) staining of xenograft tumors established with cSCC cells supported these findings, showing prominent DR4 localization at the tumor cell surface, whereas DR5, FAS, and TNFR1 were primarily localized intracellularly in vivo (Fig. 2B).

Silencing of C1s sensitizes cSCC cells to TRAIL-induced apoptosis

Silencing of C1s using specific siRNAs enhanced TRAIL-mediated apoptosis in cSCC cells (Fig. 3A, B; Supplementary Fig. S3). As residual protein C1s remained in the cell culture medium following siRNA treatment (Fig. 3A), a C1s knockout UT-SCC-7 cell line was generated. Three single-cell clones with no observed C1s production (C1s-KO) and three with remaining C1s production (KO-Ctrl) were employed to further investigate this effect (Supplementary Fig. S4; Fig. 3C, D). Complete loss of C1s significantly sensitized the cells to TRAIL-induced apoptosis (Fig. 3C, D). Additionally, C1s deficiency not only increased apoptotic signaling but also accelerated its onset, as shown by earlier activation of apoptotic signaling (Supplementary Fig. S5).

Patient-derived cSCC cell lines with low endogenous C1s are more sensitive to TRAIL -induced apoptosis

To determine whether endogenous C1s levels influence sensitivity to TRAIL-induced apoptosis, we analyzed a panel of patient-derived cSCC cell lines expressing high (UT-SCC-59A), intermediate (UT-SCC-12A), or low (UT-SCC-105) levels of C1s (Fig. 4A, B). The cSCC cell line with low endogenous C1s expression (UT-SCC-105) exhibited greater sensitivity to TRAIL-induced apoptosis compared to the high-C1s-expressing cSCC cell line (UT-SCC-59A) (Fig. 4B).

Overexpression of C1s protects cSCC cells from TRAIL-induced apoptosis

Given that low endogenous levels, siRNA-mediated silencing, and genetic knockout of C1s sensitize cSCC cells to TRAIL-induced apoptosis, we investigated whether overexpression of C1s in cSCC cells could confer resistance to TRAIL. The cSCC cell line (UT-SCC-118) with low endogenous C1s expression was transfected with C1s expression vector C1s-pCDNA3.1 (Fig. 4C, D). C1s-overexpressing (UT-SCC-$118^{C1s\ high}$) cells exhibited significantly reduced sensitivity to TRAIL-induced apoptosis compared to vector control cells (UT-SCC-118^{Ctrl}) cells (Fig. 4D). Nevertheless, dose-dependent apoptosis remained detectable in the UT-SCC-118^{Ctrl} cells (Supplementary Fig. S6).

High C1s expression in cSCC xenografts and patient samples correlates with reduced DR4 on cell surface localization

To assess the correlation between C1s expression and DR4 localization in vivo, IHC analysis was performed on two patient-derived cSCC tissue samples and cSCC xenograft tumors. In the xenograft model, regional variation in C1s expression was observed (Fig. 4E). In areas with high C1s expression, DR4 was predominantly localized intracellularly, whereas in regions with low C1s expression, DR4 was primarily detected at the cell surface (Fig. 4E). Similar IHC staining patterns were observed in two patient-derived tumor samples showing either high or low C1s expression: cell surface localization of DR4 was evident in areas with low C1s expression, while high C1s expression was associated with the absence of DR4 from the cell surface (Fig. 4E). These preliminary findings support an inverse relationship between C1s expression and DR4 cell surface localization in vivo.

TRAIL-induced apoptosis in cSCC cells is mediated primarily through the DR4 receptor

Since both DR4 and DR5 can bind TRAIL, their respective roles in mediating TRAIL-induced apoptosis in cSCC cells were investigated. Silencing of DR4 significantly reduced TRAIL-induced cell death in UT-SCC-105, which expresses low levels of C1s (Supplementary Fig. S7A, C, D), without affecting DR5 protein levels (Supplementary Fig. S7A). In contrast, silencing of DR5 had no effect on TRAIL-mediated apoptosis in these cells (Supplementary Fig. S7B, E, F) and did not alter DR4 expression (Supplementary Fig. S7B). These findings indicate that TRAIL-induced apoptosis in UT-SCC-105 cells is primarily mediated through DR4.

DISCUSSION

Recent studies have highlighted the non-canonical role of the complement system in cancer progression [17, 18]. Components of the alternative and classical pathways, such as C3, FB, FD, C1r, C1s, and C1q, along with complement inhibitors FH and FI and the receptor C5aR1, have been shown to promote the progression of cSCC, at least partly in non-canonical manner [16, 20–25]. Our previous research has shown that tumor cells in cSCC exhibit

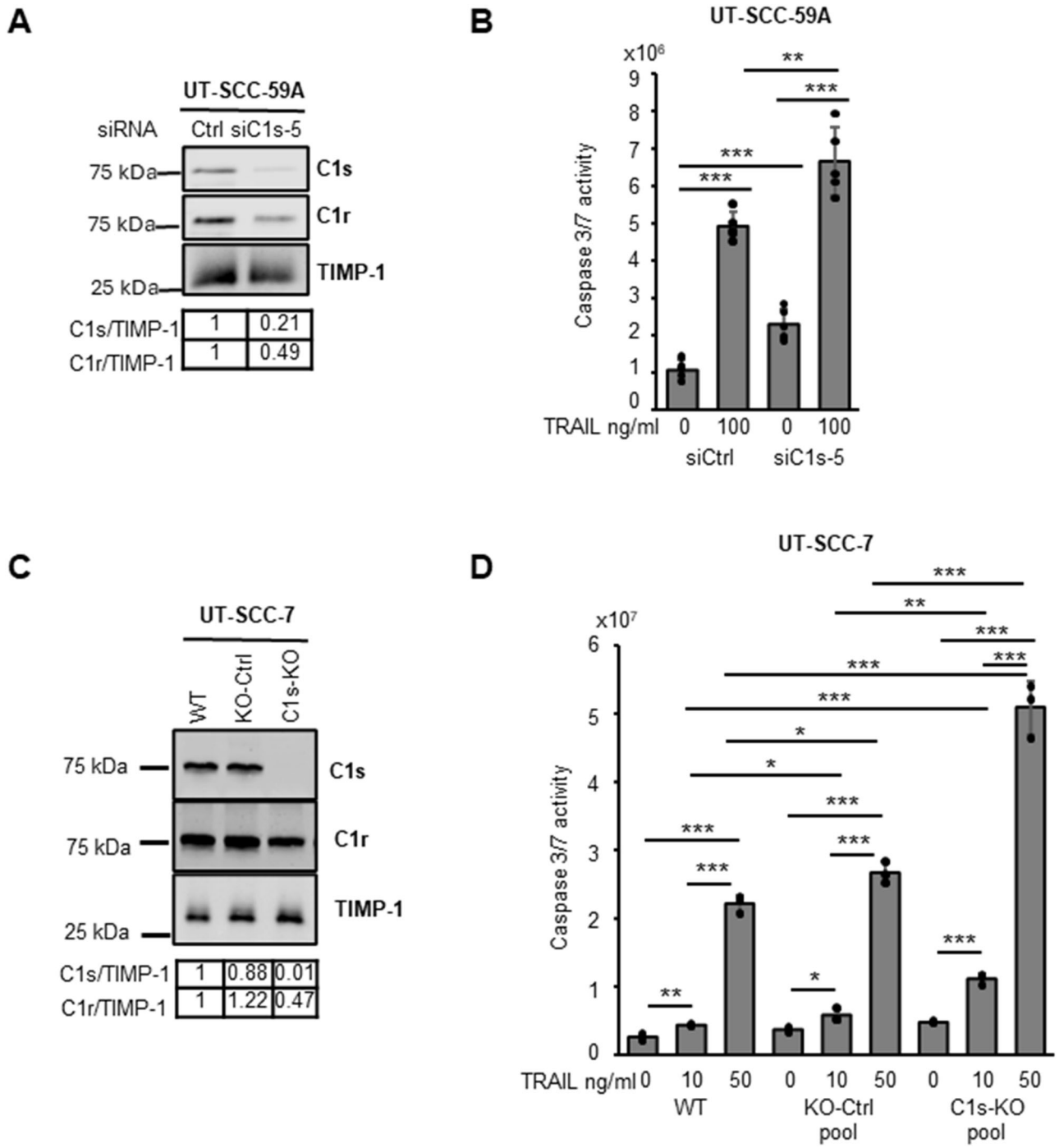

Fig. 3 Absence of C1s sensitizes cSCC cells to TRAIL-induced apoptotic signaling. A, **B** cSCC cells (UT-SCC-59A) were transfected with C1s-specific siRNA (siC1s-5) or control siRNA (siCtrl) for 24 h. **A** Western blot analysis of C1s and C1r levels in the media of siRNA-transfected cells, normalized to TIMP-1 levels in the same samples. **B** Following siRNA transfection, cells were treated with 100 ng/ml TRAIL for 20 h. Apoptotic signaling was monitored using the Incucyte® Caspase-3/7 Dye for Apoptosis. The graph displays integrated fluorescence intensity normalized to cell confluence. **C** The levels of C1s and C1r were determined by western blotting in the media of C1s knockout (C1s-KO), KO control (KO-ctrl), and wild-type (WT) UT-SCC-7 cells, and corrected to TIMP-1 levels in the same samples. In these experiments, three UT-SCC-7 C1s-KO cell clones lacking C1s production and three cell lines with residual C1s production (KO-Ctrl) were pooled. **D** C1s-KO, KO-ctrl, and WT UT-SCC-7 cells were treated with 10 or 50 ng/ml TRAIL for 15 h. Apoptotic signaling was assessed using Incucyte® Caspase-3/7 Dye, and integrated fluorescence intensity was normalized to cell confluence over time. Data are presented as mean ± s.d. with individual data points. Two-sided Student's t-test. *$p < 0.05$, ***$p < 0.01$, ***$p < 0.001$.

upregulated expression of classical pathway components C1r and C1s, while stromal fibroblasts and macrophages in cSCC show increased expression of C1q [16, 25]. Notably, knockdown of C1s or its inhibition with function-blocking antibodies has been observed to reduce cSCC growth by inducing apoptosis both in vitro and in vivo by non-canonical manner [16, 19].

In this study, we investigated how the serine proteinase C1s modifies cSCC cell surface proteins to elucidate its mechanism in

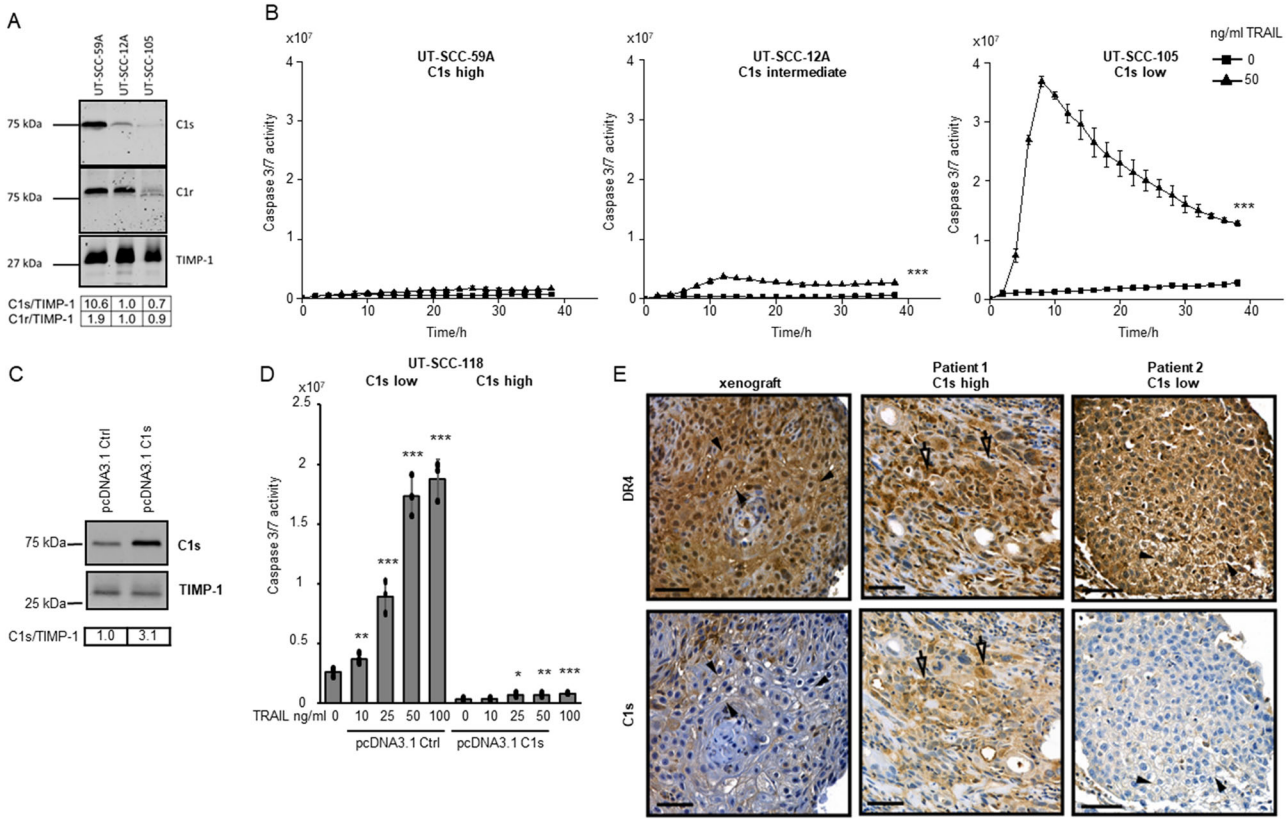

Fig. 4 Elevated C1s production protects cSCC cells from TRAIL-induced apoptosis. A Western blot analysis of C1s and C1r levels in conditioned media from various cSCC cell lines. Protein levels were standardized against TIMP-1 levels in the same samples. **B** Different cSCC cell lines with high (UT-SCC-59A), intermediate (UT-SCC-12A), and low (UT-SCC-105) endogenous C1s levels were treated with 50 ng/ml TRAIL for 24 h after plating. Apoptotic signaling was determined using Incucyte® Caspase-3/7 Dye for Apoptosis. The graph illustrates integrated fluorescence intensity over time, normalized against cell confluence at each time point (apoptotic signaling). $n = 3$. **C**, **D** cSCC cells (UT-SCC-118) were transfected with either a C1s expression vector (pcDNA3.1 C1s) or an empty control vector (pcDNA3.1 Ctrl). Positive cell pools were selected using neomycin. **C** Western blot analysis of secreted C1s levels compared to TIMP-1. **D** Cells were treated with the indicated TRAIL concentrations for 20 h, and apoptotic signaling was monitored as in (**B**). **E** Co-expression of DR4 and C1s was examined in vivo in cSCC xenografts and human cSCC tissue samples. Human metastatic cSCC cells (UT-SCC-7) were injected subcutaneously into the dorsal skin of SCID/SCID mice, and tumors were harvested after 18 days. DR4 and C1s expression was determined by immunohistochemistry. In xenografts, DR4 localized to the surface of cSCC cells in areas with low C1s expression (left panels, black arrows). In human cSCC tumors, DR4 expression was cytoplasmic in areas with C1s expression (patient 1; middle panels; white arrows) and localized to the tumor cell surface in areas lacking C1s expression (patient 2; right panels, black arrows) in vivo. Scale bar = 50 μm. Data are presented as mean ± s.d. with individual data points. Two-sided Student's t-test. *$p < 0.00$, ***$p < 0.01$, ***$p < 0.001$.

evading tumor cell apoptosis. Mass spectrometric analysis of cSCC cell surface proteins was conducted following C1s silencing to identify potential direct or indirect target molecules for C1s. Interestingly, apoptotic signaling-inducing receptor DR4 was found to accumulate on the cSCC cell surface following C1s silencing, despite no regulation being observed in DR4 mRNA levels. This finding confirms that only the protein levels on the cell surface are regulated, which could be a mechanism by which C1s regulates cSCC cell growth. Previous studies have demonstrated that elevated DR4 expression is associated with increased sensitivity to TRAIL, whereas reduced expression levels may contribute to resistance to apoptotic signaling [26].

DR-induced pathways are important for apoptosis triggered by cytotoxic T-lymphocytes and natural killer cells during immune responses [27]. Previous studies have highlighted the involvement of DRs in cancer progression [28]. For example, tissue inhibitor of metalloproteinases-3 (TIMP-3) induces apoptosis in melanoma cells by stabilizing death receptors on their surface [29, 30]. DR4 is among the DRs that induce apoptosis in p53-transformed cancer cells [28, 31]. Interestingly, UV-induced cSCC carries a high burden of mutations, with the *TP53* gene being mutated in 50–60% of primary tumors and nearly 95% of metastatic cSCCs [5–7]. This suggests that cSCC could be a promising target for TRAIL-based therapeutic strategies [32].

In our study, all cSCC cell lines examined had at least one mutated TP53 allele [33, 34]. Of other DRs involved in the regulation of apoptosis, FAS was identified in mass spectrometric analysis. However, its expression on the surface of cSCC cells remained unchanged after C1s silencing. Neither DR5 (TRAIL-R2) nor TNFR1 was detected in the mass spectrometric analysis of cSCC cells. In this study, the levels of all these DRs on surface of cSCC cells were confirmed by western blotting in cultured cells and IHC in vivo. The results showed that the only DR to accumulate on the cSCC cell surface in vivo was DR4, suggesting it as the primary TRAIL-binding receptor and a direct or indirect target of C1s in cSCC. C1s has been reported to cleave specific non-canonical proteins in the extracellular space or at the cell surface, potentially promoting cell proliferation or survival [35]. Additionally, our previous studies have shown that the related classical pathway serine protease C1r regulates matrix metalloproteinase-13 expression in cSCC [15]. Based on our findings, C1s may directly cleave DR4. However, we cannot exclude an indirect mechanism, such as C1s-mediated increase of matrix metalloproteinase expression and activity.

Previous studies have demonstrated the role of TRAIL and DR4 in activating caspases and apoptosis in transformed keratinocytes [36, 37]. To explore the impact of TRAIL-induced apoptosis on cSCC cells with varying C1s levels, TRAIL-induced caspase activity was measured. Our findings indicate that absence or low levels of C1s sensitize cSCC cells to TRAIL-induced apoptosis. Therefore, increased C1s expression during cSCC progression might be a strategy for cSCC cells to evade apoptosis. Further investigation using specific siRNAs revealed that knockdown of DR4, but not DR5, affects apoptotic signaling upon TRAIL addition, indicating that DR4 is the functional DR in cSCC cells in culture.

Cancer cells develop resistance mechanism to evade TRAIL-induced apoptosis [38]. DR4 is particularly important in cancer therapy because it can selectively trigger cell death in malignant cells without affecting normal cells [39]. This selective effect makes targeting TRAIL signaling a widely investigated approach in cancer treatment, highlighting its potential as an ideal therapeutic agent for tumor cells [32]. Furthermore, complement components, including C1s, are upregulated in several cancer types, contributing to cancer development [18]. Drugs initially developed for complement-related diseases could potentially be repurposed for cancer treatment [40]. Notably, function-blocking antibodies targeting C1s have been shown to inhibit growth of cSCC cells [19]. Moreover, the humanized form of these C1s antibodies, sutimlimab, has been approved by the FDA and EMA for the treatment of cold agglutinin disease (CAD), indicating potential applications in other diseases [41].

The findings of this study emphasize the crucial role of C1s in cancer progression through its regulation of TRAIL-induced apoptosis in cSCC cells. Inhibiting C1s could therefore represent a promising therapeutic strategy. Several strategies to enhance the apoptotic efficacy of TRAIL have been explored, including combining TRAIL with sensitizers such as phloretin or thymoquinone, which have been shown to induce a more robust apoptotic response in cancer cells [42]. A dual-targeting approach, involving both C1s and DR4, could be particularly effective. We propose that initially blocking C1s function would stabilize DR4 on the cell surface, thereby sensitizing cSCC tumor cells to TRAIL-induced apoptosis and promoting efficient cancer cell killing.

MATERIALS AND METHODS

Ethical issues

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Turku University Hospital (187/2006; Approval date: 26 April 2006). Informed consent was obtained from all subjects involved in the study. Permission for tissue samples has been granted by Auria Biobank (AB15-9721) and Turku University Hospital Clinical Research Centre (T80/2018). Mouse experiments were approved by the National Animal Test Review Board of the Government of the Region Southern Finland (ESAVI15107/2020). All mouse experiments were performed according to the institutional guidelines.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [43] partner repository with the dataset identifier PXD065511 (Project accession: PXD065511).

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COMPETING INTERESTS

The authors declare no competing interests.

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